INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

INVESTIGATION OF MACROLICHENS FOR ANTIFUNGAL POTENTIALITY AGAINST PHYTOPATHOGENS

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ARTICLE INFO

Article history
Received 20/04/2016
Available online
30/04/2016

Keywords
Lichen,
Phytochemicals,
Ramalina Farinacea,
Parmotrema Margaritatum,
Fusarium,
Bioautography.

ABSTRACT

In the present study fungicidal potential of four macrolichens *Heterodermia comosa*, *Parmotrema margaritatum*, *Pseudocyphellaria aurata* and *Ramalina farinacea* were evaluated against plant pathogenic fungi *Fusarium oxysporum* and *Fusarium solani*. The hot crude extract was extracted from solvent methanol, ethyl acetate and acetone. The highest yield of 157 mg/ml was obtained from methanol extract of *Pseudocyphellaria aurata.* Important phytochemical constituents like tannins, alkaloids, carbohydrates and steroids were present in most of the lichen extracts tested. Antifungal activity of the extracts was evaluated by well diffusion assay, micro dilution assay and TLC bioautographic technique. The ethyl acetate extract of *Ramalina farinacea* showed highest zone of inhibition against *Fusarium oxysporum* with 17.0±1.0 mm and *Fusarium solani* with 13.3±0.57 mm. Micro dilution assay showed least MIC value of 0.781 mg/ml from *Ramalina farinacea* and *Heterodermia comosa* in ethyl acetate extracts against *Fusarium oxysporum* and 3.125 mg/ml against *Fusarium solani.* Clear zones of fungal inhibition were obtained on chromatogram in the ethyl acetate extract of *Parmotrema margaritatum* at R_f value 0.1, 0.21, 0.48. The obtained results prove that lichens can be used as potential antifungal agents against phytopathogens. Hence lichens serves as better natural fungicides in order to keep the environment safe, and which may also increase the yield and production of the crops. Further work could be focused on the isolation and purification of active components from the studied lichens.

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*Please cite this article in press as **Rashmi Shivanna** et al. Investigation of Macrolichens for Antifungal Potentiality Against Phytopathogens. Indo American Journal of Pharmaceutical Research.2016:6(04).*

INTRODUCTION

Plant diseases, in particular caused by fungal pathogens, pose a major threat to economically valuable crops in the field and also post-harvest, thereby decreasing production and shelf life of many agricultural crops [1]. Different approaches are used to prevent, mitigate or control plant diseases. The most important method of protecting plants against fungal attack is the use of fungicides. A large number of chemical fungicides that are used to control these pathogenic fungi are toxic and have undesirable effects on other organisms present in the environment and therefore there is a need to replace by safe, biodegradable products [2]. One such natural product could be lichens. Lichens are symbiotic organisms composed of fungi and algae, it is estimated that there are about 20,000 species of lichens present throughout the world [3]. The photosynthetic partner (algae) provides nutrient to the fungus while the fungus provides the structure that protects the photosynthetic partner from drying [4]. Lichens produce a diverse range of primary and secondary metabolites that were used as medicines, cosmetics, dyes, foods and decorations [5]. The specific, even extreme, conditions of their existence, slow growth, and long duration (maximum lifetime spans to several thousand years) are consistent with their abundance in protective metabolites against different physical and biological influences [6]. Lichen forming fungi produce antibiotic secondary metabolites that protect many animals from pathogenic microorganisms [7]. In contrast, secondary metabolites are produced exclusively by the fungal partner and are exported outside the fungal hyphae and deposited as crystals in different parts of the thallus, often in the upper cortex or in specialized structures such as fruiting bodies [8].

Lichen metabolites exert a wide variety of biological actions including antimycotic, antibacterial, antiviral, anti-inflammatory, antioxidant, antidiabetic, antiobesity, analgesic, antipyretic, antiproliferative and cytotoxic effects [9]. Lichen secondary metabolites have been investigated mostly for chemotaxonomic purposes and in connection with their potential as phytomedicines and natural biopesticides [10]. The bioactive nature of lichen secondary compounds suggests possible strategies to manage plant pathogens, which may include interference with different developmental stages, such as prevention of spore germination and inhibition of sexual reproduction, or interference directly with mycelial growth. These strategies are perhaps achievable with the application of a purified bioactive compound or a suite of components with a synergistic effect [11, 12]. Even though manifold activities of lichen metabolites have now been recognized their therapeutic potential has yet not been fully explored and thus remains pharmaceutically unexploited [13]. The purpose of the present work was to conduct *in vitro* evaluation of the antifungal metabolites manifested by methanol, ethyl acetate and acetone extracts from four species of lichens (*Heterodermia comosa*, *Parmotrema margaritatum*, *Pseudocyphellaria aurata* and *Ramalina farinacea*) in relation to agents causing plant diseases.

MATERIALS AND METHODS

Collection and Identification of lichens

The lichen specimen was collected from different regions from Madikeri and Chamarajnagar districts in Karnataka. Lichens were identified by morphological, anatomical and chemical tests [14, 15] as *Heterodermia comosa* (Eschw.) Follmann & Redón, *Parmotrema margaritatum* (Hue) Hale, *Pseudocyphelaria aurata* (Ach.) Vainio and *Ramalina farinacea* (L.) Ach. The collected lichens were preserved in the herbarium of the Department of studies in Botany, Manasagangotri, University of Mysore, Karnataka, India.

Processing of lichen extracts

Lichens were washed thoroughly 2-3 times with distilled water to remove all the impurities and shade dried at room temperature. The dried lichens were milled to a fine power with the help of a blender and stored at room temperature in closed containers in the dark until used. Powdered lichen samples of about 10 gms from each species were extracted by standard Soxhlet extraction procedure using Ethyl acetate, Methanol and Acetone solvents. Later it was filtered through Whatman No.1 filter paper and were concentrated by air-drying for 4 –5 days preserved at 5℃ in airtight bottles until further use [16].

Preliminary phytochemical evaluation

The different extracts thus obtained were qualitatively tested for the presence of various phytochemical constituents like tannins, alkaloids, saponins, glycosides, flavonoids, proteins, triterpenoids, carbohydrates and steroids [17, 18].

Spot test

The colour test also known as spot test. Presence of certain lichen substances in tissues of lichen thallus produces a change in their colour when certain chemicals are applied on the surface of thallus. A positive change will be denoted by a positive (+) symbol, followed by the colour produced, and no change in colour denoted by a negative (-) symbol. The chemicals routinely used as follows: K- test, C- test, KC- test, Pd- test.

Isolation of fungal strains

For the present study two *Fusarium* species were selected which causes major crop diseases. The fungus *Fusarium oxysporum* f.sp. *capsici* which is a causal organism of *Fusarium* wilt in chilli was isolated from the diseased chilli plants. And the other fungus *Fusarium solani* (Mart.) Sacc., a causal organism of rhizome rot in ginger was isolated from the diseased rhizome part of ginger by the standard blotter method and the fungi were identified on the basis of their typical structure and basic characters [19]. The isolated fungus was maintained on potato dextrose agar (Himedia) at 26-28℃. The standard culture inoculum was prepared on Potato Dextrose Broth by adjusting the spore range of 1×10^6 - 5×10^6 spores /ml [20].

Determination of Antifungal Activity

Agar well diffusion:

100 µl of fungal suspension was spread on the solidified Potato dextrose agar medium and wells were punched using 5mm cork borer, a concentration of 30 mg/ml extracts of 100 µl was loaded into the wells and the solvents of the same were used as a negative control. The plates were kept for incubation for 4-7 days; the diameter of the zone of inhibition of the tested microorganism by the given extract was measured in millimeters. All experiments were performed in triplicate. To every sample tested, a set of control was run parallel [21].

Microdilution assay:

Determination of Minimum inhibitory concentration [MIC] was carried out by microdilution method. The MIC is to determine the lowest concentration of an antifungal agent that appears to inhibit growth of the fungus. Residues of different extracts were dissolved in respected solvents to a concentration of 50mg/ml. The plant extracts (100µl) were serially diluted 50% with solvents in 96 well flat bottomed microtitre plates. Fungal cultures were transferred into fresh Potato dextrose broth, and 100µl of this was added to each well, 40µl of 2, 3, 5- triphenyltetrazolium chloride [TTC] dissolved in water was added to each of the micro plate wells, as growth indicator. Appropriate solvent blanks as control were included. The micro plates were covered with a cling film and incubated for 2-3 days at 26℃ and at 100% relative humidity [22]. The MIC was recorded by visual analysis in microtitre plate wells, where the lowest concentration of the lichen extract that inhibited fungal growth after 48 to 72 hours of incubation will not change its colour to formazen dye.

Bioautography method:

Agar overlay Bioautographic method was developed to determine active compounds. Aluminium-backed TLC plates (Aluchrosep Silica Gel 60/UV_{254} for TLC) were loaded with 20µl of 100µg extracts. The TLC plate was developed in solvent system A (180 ml toluene: 60 ml 1-4, dioxin: 8 ml acetic acid) [23]. The chromatogram was dried for complete removal of solvents. Fungal inoculum solution was prepared containing approximately 3 x 10^4 spores/ml of actively growing fungi. Developed chromatogram was placed in petridish molten potato dextrose agar seeded with fungal inoculum was poured on the chromatogram. After agar got solidified the petri plates were kept at 4^{o}C for diffusion for 3 hours and the plates were incubated at room temperature for 4 days. Fungal growth inhibition appeared as clear zones against a dark background. The R_f value of the zone of inhibition is recorded.

RESULTS AND DISCUSSION

Antifungal potentialities of some macrolichens were evaluated against phytopathogenic fungi *Fusarium oxysporum* f.sp. *capsici* and *Fusarium solani*. Lichens *Heterodermia comosa*, *Parmotrema margaritatum*, *Pseudocyphellaria aurata* and *Ramalina farinacea* were collected from Madikeri and Chamarajanagar districts of Karnataka (Table 1). In this study collected lichens were extracted with different solvents like methanol, ethyl acetate and acetone. The total mass of the yield varied from 32 to 157 mg/ml. The highest yield of 157 mg/ml was obtained from methanol extract of *Pseudocyphellaria aurata*, followed by 146 mg/ml of methanol extract of *Heterodermia comosa*, whereas lowest yield was obtained from the acetone extracts of all the lichens (Fig. 1).

Table 1: List of lichens collected from different places of South Karnataka.

Sl no.	Lichen sample	Growth form	Family	Place of collection
1	*Heterodermia comosa* (Eschw.) Follmann & Redón.	Foliose to sub fruticose	Physciaceae	Biligiriranga hills, Chamarajnagar
2	*Parmotrema margaritatum* (Hue) Hale	Foliose	Parmeliaceae	Nisargadhama, Madikeri
3	*Pseudocyphellaria aurata* (Ach.) Vainio	Foliose	Stictaceae	Dubare forest, Madikeri
4	*Ramalina farinacea* (L.) Ach.	Fruticose	Ramalinaceae	Nisargadhama, Madikeri

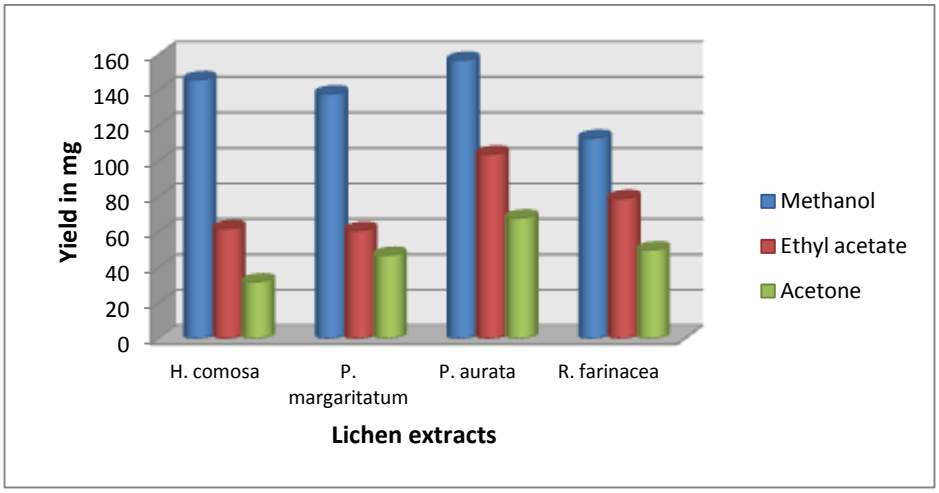

Fig 1: Total yield of lichen extracts obtained from different solvents.

Phytochemical screening for the presence of tannins, alkaloids, saponins, glycosides, flavonoids, proteins, triterpenes, carbohydrates and steroids was carried out on aforesaid extracts. Important phytochemicals like tannins, alkaloids, carbohydrates and steroids were present in most of the lichen extracts tested (Table 2). Constituents like tannins, alkaloids, cardiac glycosides, steroids, carbohydrates were present in *Heterodermia comosa*. Tannins, alkaloids, proteins, triterpenes were present in *Parmotrema margaritatum*. Saponins and steroids were present in *Pseudocephellaria aurata*, tannins, alkaloids, carbohydrates were present in *Ramalina farinacea*. Spot test showed the presence of Atranorin in *Heterodermia comosa* and *Parmotrema margaritatum* along with zeorin and salazinic acid respectively. *Pseudocyphellaria aurata* contained only calycin, whereas *Ramalina farinacea* showed more number of chemicals like usnic acid, norstictic acid and protocetraric acid (Table 3).

Table 2: Phytochemical constituents of lichen extracts.

Phytochemical constituents	**Tests**	***Heterodermia comosa***	***Parmotrema margaritatum***	***Pseudocyphelaria aurata***	***Ramalina farinacea***
Tannins	$FeCl_3$ test	+	+	-	+
Alkaloids	Dragondroff's test	+	+	-	+
Saponins	Frothing test	-	-	+	-
Cardiac glycosides	Keller-kiliani test	+	-	-	-
Steroids	Liebermann-Burchard reaction	+	-	+	-
Flavanoids	NaOH solution test	-	-	-	-
Proteins	Xanthoproteic test	-	+	-	-
Triterpenes	Salkowski test	-	+	-	-
Carbohydrates		+	-	-	+

+: Presence of compound, - : Absence of compound

Table 3: Spot test of lichen thallus.

Sl no.	**Lichen sample**	**Spot test**	**Lichen substances**
1	*Heterodermia comosa* (Eschw.) Follmann & Redón.	K+ yellow, C- KC- ,P+ yellow	Atranorin, Zeorin
2	*Parmotrema margaritatum* (Hue) Hale	K+red, C- KC- P-	Atranorin, Salazinic acid
3	*Pseudocyphellaria aurata* (Ach.) Vainio	K- C-KC- P-	Calycin
4	*Ramalina farinacea* (L.) Ach.	K-C-KC+ yellow, P+ yellow orange	Usnic acid, Norstictic acid, Protocetraric acid

The well diffusion assay was carried out to test the zone of inhibition against the tested fungi which ranged from 11.3±0.57 to 17.0±1.0 mm in *Fusarium oxysporum* and 11.6±0.57 to 13.33±1.15 mm in *Fusarium solani* (Table 4 and Table 5). Lichen extracts showed maximum activity in the ethyl acetate extract, followed by methanol extract. Acetone extracts did not show any activity. The ethyl acetate extract of *Ramalina farinacea* showed highest activity on *Fusarium oxysporum* with 17.0±1.0 mm zone of inhibition and 13.3±0.57 mm against *Fusarium solani*. For methanolic extract highest activity was recorded in *Ramalina farinacea* by 16.6±1.15 mm. Rest of the extracts showed moderate activity 16.0±0, 15.3±0.57 and 14.6±1.15 mm in *Heterodermia leucomela*, *Pseudocephellaria aurata* and *Parmotrema margaritatum* respectively against *Fusarium oxysporum*.

Minimum inhibitory activity was tested for the lichen extracts which showed positive results in diffusion assay. Micro dilution assay showed least MIC value of 0.781 mg/ml from *Ramalina farinacea* and *Heterodermia comosa* in ethyl acetate extract against *Fusarium oxysporum* and 3.125 mg/ml against *Fusarium solani*. The next lowest concentration of 1.562 mg/ml was recorded in the methanol extract of other lichens. Antifungal metabolites were identified by TLC bioautography. Fungal growth inhibition was observed as clear zones on the chromatogram. 3 spots of inhibition zones were observed in ethyl acetate extract of *Parmotrema margaritatum* at R_f value 0.1, 0.21, 0.48. Methanol and ethyl acetate extract of *Ramalina farinacea* showed 2 spots each of inhibition zones at R_f value 0.32, 0.6 and 0.64, 0.82 respectively against *Fusarium oxysporum*. Only ethyl acetate extract of *Parmotrema margaritatum* showed inhibition zone on chromatogram against *Fusarium solani* at R_f value 0.46 and 0.6.

Table 4: Antifungal activity of lichen extracts on phytopathogenic fungi *Fusarium oxysporum.*

Sl no.	Lichen sample	Solvents	Disc diffusion (in mm)	MIC (in mg/ml)	Bioautography (R_f value)
1	*Heterodermia comosa* (Eschw.) Follmann & Redón.	Methanol	11.3±0.57	1.562	0.89
		Ethyl acetate	16.0±0	0.781	0.86, 0.71
		Acetone	-	-	-
2	*Parmotrema margaritatum* (Hue) Hale	Methanol	12.6±1.15	1.562	0.65
		Ethyl acetate	14.6±1.15	3.125	0.1, 0.21, 0.48
		Acetone	-	-	-
3	*Pseudocyphellaria aurata* (Ach.) Vainio	Methanol	11.3±0.57	1.562	-
		Ethyl acetate	15.3±0.57	3.125	0.35
		Acetone	-	-	-
4	*Ramalina farinacea* (L.) Ach.	Methanol	16.6±1.15	3.125	0.32, 0.6
		Ethyl acetate	17.0±1.0	0.781	0.64, 0.82
		Acetone	-	-	-
5	Pure Solvent (negative control)	Methanol	-	-	-
		Ethyl acetate	-	-	-
		Acetone	-	-	-
6	Bavistin (positive control)	-	18.5±0.3	0.390	-

- : No activity

Table 5: Antifungal activity of lichen extracts on phytopathogenic fungi *Fusarium solani.*

Sl no.	Lichen sample	Solvents	Disc diffusion (in mm)	MIC (in mg/ml)	Bioautography (R_f value)
1	*Heterodermia comosa* (Eschw.) Follmann & Redón.	Methanol	-	-	-
		Ethyl acetate	11.6±0.57	3.125	-
		Acetone	-	-	-
2	*Parmotrema margaritatum* (Hue) Hale	Methanol	-	-	-
		Ethyl acetate	13.33±1.15	6.25	0.46, 0.6
		Acetone	-	-	-
3	*Pseudocyphelaria aurata* (Ach.) Vainio	Methanol	-	-	-
		Ethyl acetate	12.0±1.0	6.25	-
		Acetone	-	-	-
4	*Ramalina farinacea* (L.) Ach.	Methanol	-	-	-
		Ethyl acetate	13.3±0.57	3.125	-
		Acetone	-	-	-
5	Pure Solvent (negative control)	Methanol	-	-	-
		Ethyl acetate	-	-	-
		Acetone	-	-	-
6	Bavistin (positive control)	-	17.3±0.3	0.781	-

- : No activity.

The lichen extracts which was tested against the *Fusarium* sp. showed relatively strong antifungal activity. From our results comparatively *Ramalina farinacea* showed a remarkable activity in both ethyl acetate and methanol extracts against *Fusarium oxysporum* and *Fusarium solani*. Extracts of lichens *Heterodermia comosa*, *Parmotrema margaritatum* and *Psuedocephallaria aurata* manifested moderate inhibitory activity against the tested fungi. Similar results were obtained in a study of the antifungal activity of lichen extracts (including extracts of the species *Hypogymnia physodes*) against phytopathogenic fungi [24]. Different extracts of lichen thalli *Ramalina farinacea* tested proved to have strong other biological activity like antiviral, antimicrobial, antibacterial and cytotoxic [25, 26, 27, 28, 29]. Preliminary phytochemical tests showed the presence of tannins, alkaloids, carbohydrates and lichenic substances like usnic acid, norstictic acid, protocetraric acid were detected by the spot test and TLC in *Ramalina farinacea*. In our previous research, several macrolichens have showed promising antifungal properties [30].

CONCLUSION

From the present study, obtained results proves that lichens can be used for the discovery and development of new chemical substances to control fungal diseases which is greatly needed and has promoted studies of traditionally used natural products, which are generally considered to be very important sources of bioactive substances. This suggests the advantageous for isolation of the active components from the extracts of the investigated lichen for possible use in treating many diseases caused by various microorganisms.

ACKNOWLEDGEMENT

The authors are thankful to University of Mysore for awarding UGC NON-NET fellowship to the first author.

CONFLICT OF INTEREST NOTIFICATION PAGE

The authors have no conflict of interest to declare.

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